AMENDMENTS TO THE SPECIFICATION

Please replace the specification with the substitute specification submitted with markings showing all the changes relative to the prior version of the specification of record. Also included is a clean version (without markings). Applicants submit that the amendments to the specification are supported by the specification and claims as originally filed and do not introduce new matter.

Clean version of substitute specification

A recombinant fusion protein, a (vaccine) substance composition containing it and a method for the preparation thereof

[0001] The invention relates to a recombinant fusion protein, a (vaccine) substance composition containing the recombinant fusion protein, and a method for the preparation of the recombinant fusion protein.

[0002] The edema of pigs is caused by Shiga toxin forming *Escherichia coli* (STEC). The main virulence factor of these pathogenic organisms which is exclusively accountable for the clinical symptoms is the 2e Shiga toxin (Stx2e) (Mac Leod et al., 1991). Since the disease exhibits a peracute progress in many cases and attempts for a therapy mostly are initiated too late or do not result in the success desired it would be desirable to develop an efficient prophylaxis. It is problematic to produce and thoroughly purify the Stx2e.

[0003] The B sub-unit of the Stx2e is taken into account as a possible vaccine for various reasons. It is identified by the serums of convalescent piglets, i.e. it possesses antigenic determinants. In addition, the B sub-unit of the toxin induces the formation of toxin-neutralizing antibodies after a parenteral application (Acheson et al., 1996; Boyd et al., 1991). Genetic engineering methods were a successful aid in preparing a recombinant fusion protein which consists of a fragment of the Stx2eB sub-unit and the Glutathion S transferase of Shistosoma Japonicum (Franke et al., 1995). For the edema of weaned piglets, both the excretion of the pathogenic organisms and the immunological reaction to the STEC infection was investigated already over a major period of time. The recombinant fusion protein from a fragment of the Stx2eB sub-unit and the Glutathion S transferase, which was used to prove the presence of Stx2e antibodies, is suited very well to indirectly prove the STEC infection and has been hitherto considered to be a potential vaccination antigen for the prophylaxis of the edema (Wieler L. H., Franke, Sylvia, Rose M., and Karch, H.: Charakterisierung der Immunantwort bei der Odemkrankheit des Schweines mit einer rekombinanten B-Untereinheit des Shiga-like-Toxins II_e, (Lecture read at the 21st DVG congress at Bad Nauheim (in March, 1995)).

[0004] Therefore, it is the object of the invention to provide a recombinant fusion protein suited for vaccination purposes, a plasmid encoding it, a (vaccine) substance composition containing the fusion protein for various applications in conjunction with the edema, particularly that of the pigs, and a method for the preparation of the recombinant fusion protein.

[0005] The object is achieved by a recombinant fusion protein having the features of claim 1, a (vaccine) substance composition having the features of claim 5, an *E. coli* strain according to the plasmid according to claim 18, and a method having the features of claim 20. Aspects of the invention are indicated in the sub-claims.

[0006] According to the invention, a recombinant fusion protein and a (vaccine) substance composition containing it are provided which may be used for various applications in conjunction with the edema, particularly that of the pigs. Thus, the applications taken into consideration are:

- The demonstration of antibodies against Stx2e,
- the diagnosis of the edema,
- the generation of monoclonal antibodies against the toxin of the pathogenic organism causing the edema, specifically as a basis of checking the yield in deriving the recombinant fusion protein or as a basis of deriving the holotoxin by immune affinity chromatographic purification,
- The immunization against the edema, particularly that of the pigs.

[0007] The recombinant fusion protein is a Stx2e fragment of the 2e Shiga toxin in a fusion with a terminal tag the size of which approximately corresponds to the size of the fragment or a fraction of the fragment. The terminal tag is a marked end group in the amino-acid sequence of the protein. Preferably, the Stx2e fragment is a B sub-unit (Stx2eB) of the 2e Shiga toxin. The size of the terminal tag is preferably 5 kDa, as a maximum, and more preferably is 5 kDa. Also preferably, it is an amino terminal His tag. The His tag comprises six histidines. Its size is about 0.66 kDa.

[0008] The recombinant fusion protein has substantial antigenic domains of the native protein which substantiate its suitability for various applications in conjunction with the edema. It is true that this has also been the theoretical case for the previously known recombinant fusion proteins from a fragment of the Stx2eB sub-unit and the Glutathion S transferase. However, the problem posed here is that as the applicant judges it annoying, immunological reactions have to be expected that oppose the use of the generic fusion proteins for therapeutic applications. In contrast, a significant advantage of the inventive fusion protein is that annoying immunological responses are not expected here because of the tag which is especially chosen and, thus, for the first time, fusion proteins will be available that are usable in vaccines. Like for generic fusion proteins, the tag used according to the invention facilitates the derivation of the recombinant fusion protein, particularly its purification, e.g. by an affinity chromatographic method.

[0009] Oligomers from crosslinked His Stx2eB monomers may form fusion proteins which are particularly efficient.

[0010] According to an advantageous aspect, the (vaccine) substance composition, in addition to the recombinant fusion proteins, comprises at least one additional antigen. A vaccine substance composition is a formulation of an immunogenic amount of the recombinant fusion protein and an immunogenic amount of at least one additional antigen. This combined vaccine is apt to effect a simultaneous vaccination against the edema of the pigs and against at least one further disease.

[0011] In particular, the (vaccine) substance composition, in addition to the recombinant fusion proteins, may comprise at least one additional antigen which is selected from the group comprising: a Pasteurella multocida bacterin including a cell-bonded toxoid, a Bordetella bronchiseptica bacterin, an Erysipelothrix rhusiopathiae antigen, one or more soluble non-cell toxoids of type D Pasteurella multocida and/or Escherichia coli and/or Clostridium perfringens, inactivated

whole cells of type A or D Pasteurella multocida, cultures of Actinobacillus pleuropneumoniae, Haemophilus parasuis, Escherichia coli, Clostridium perfringens, Streptococcus suis, Mycoplasma hyopneumoniae as well as Porcine Reproduction and Respiratory Syndrome virus, influenza virus, Pseudorabies virus, and Porcine Circoviruses I and II.

[0012] The aforementioned antigens are known to cause the diseases which follow: Pasteurella multocida and Bordetella brochiseptica cause the progressive atrophic rhinitis of the pigs, also called "snuffle disease"; in a pathogenic respect, it is mainly the Pasteurella multocida toxins which play an important part (with the toxoid content being significant in commercial vaccines). Pasteurella A and D occur in respiratory diseases of the pigs (pneumonia). The Pasteurella multocida D also causes the snuffle disease. The Erysipelothrix rhusopathiae causes pig erysipelas.

[0013] The *Escherichia coli* causes diarrhoea diseases (where the edema of the pigs is a special form) (the toxins are decisive). The Clostridium perfringens causes the necrotizing enteritis of the suckling piglets (the toxins are decisive). The Actinobacillus pleuropneumoniae causes hemorrhagic necrotizing pleuropneumonia.

[0014] The Haemophilus parasuis causes the Glasser disease (fibrinous serositis and arthritis). The Streptococcus suis causes streptococcal septicaemia. The Mycoplasma hyopneumoniae causes enzootic pneumonia, also called "Piglet influenza."

[0015] The Porcine Reproductive and Respiratory Syndrome virus causes respiratory diseases (pneumonia) of piglets and fertility diseases of sows. The influenza virus causes respiratory diseases.

[0016] The Pseudorabies virus causes the Aujeszky disease of the pig (pseudo-rage).

[0017] The Porcine Circoviruses I and II-causes the post-weaning multisystemic wasting syndrome.

[0018] Preferably, at least one additional antigen is chosen so as to refer to a disease which typically attacks the pig at approximately the same age as the edema does. This is largely the case for the above mentioned antigens. The vaccine substance composition will then make possible a particularly operative combined vaccination.

[0019] The vaccine composition preferably contains the recombinant fusion protein and/or at least one additional antigen each in an immunogenic amount for the vaccination of pigs against the edema of the pigs and other viral and/or bacterial infections.

[0020] In addition, the invention relates to vaccine substance compositions the compositions and/or amounts of which are chosen so as to make achievable an immunization of the animal concerned against at least one disease by sequential and/or simultaneous vaccination with the vaccine compositions.

[0021] The choice of the adjuvant is of particular significance for the vaccine (substance) composition. For instance, a W/O/W emulsion (e.g. ISA 206), a W/O emulsion (e.g. an iFA incomplete Freund adjuvant, an aqueous suspension (e.g. aluminum hydroxide) or an O/W emulsion may be employed.

[0022] According to the inventive method for the recombinant preparation of a fragment of the 2e Shiga toxin (Stx2e) in a fusion with a terminal tag, a suitable vector system of a sub-unit is cloned from the Stx2e operon, the resultant recombinant plasmid is transformed into an *E. coli* strain, the resultant expression system is induced, and the fusion protein is expressed and purified.

[0023] The gene of the B sub-unit of the 2e Shiga toxin (Stx2eB) was cloned into various expression vectors. The recombinant plasmids thus formed were used for transforming various *E. coli* K12 laboratory-scale strains. All transformants were tested under varying conditions (temperature, level of induction, duration of

induction) in expression studies for the formation of the recombinant B sub-unit. The transformant or clone having the largest yield of recombinant protein as compared to the cell protein overall content was determined. A purification method was developed for the fusion protein formed in this strain, comprising the mature B sub-unit with an N terminal His tag (His-Stx2eB), and was tested at a laboratory scale. FPLC which uses appropriate buffer systems is contemplated for implementing the purification method at a large scale.

Example:

Preparation of the recombinant B sub-unit of the Stx2e

[0024] The strain *E. coli* Cox-Stx2eB, DSM No. 12721 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124 Braunschweig) is used for the preparation of the recombinant B sub-unit. This *E. coli* laboratory-scale strain contains the plasmid pHIT-24 which clones the B sub-unit of the Stx2e.

[0025] A seed lot system was set up from this strain, was filled into 2 ml cryo vials, and was stored at -78 °C.

[0026] For the production of the recombinant B sub-unit, 1 ampulla of working seed (2 ml) is defrosted for the growth of a pre-culture 1. The pre-culture 1 is prepared under the following conditions: Medium: 150 ml sterile standard I nutrient broth + 0.01 % Ampicillin in a 300 ml Erlenmeyer flask

Incubation: for 15 hours at 37 °C, upright stationary culture

[0027] A "Biostat B" fermenter having a 5-litre culture vessel is used to prepare the main substance. This vessel is filled with 4 litres of standard I nutrient broth + 0.01 % Ampicillin and was autoclaved as a unit for 25 minutes at 121 °C. The pre-culture 1 is placed in this medium and is cultivated for 6 hours under the following conditions:

Temperature: 37 °C

pH = 7.0 to 7.1

Stirring speed: from 100 to 150 rpm

Air supply: 2 litres/min

[0028] The regulation of the pH is ensured by an automatic feed of a sterile 10 % NaOH solution.

[0029] Induction was initiated by adding 0.25 mM of an IPTG solution* after a cultivation of 6 hours and a pH leap from 7.1 to 7.5. The induction period was ~~abt.~~ about 3.5 hours.

[0030] Subsequently, the culture was pumped into a 10-litre harvesting container and was hydroextrated in a centrifuge of 2500 x g. The supernatant substance was discarded, the pellet was received in 200 ml of an 8 M urea buffer and was kept in a refrigeration room (at 4 to 8 °C) for about 15 hours. The resuspended pellet was then treated with ultrasonic sound (for 4 x 15 minutes at 190 Hertz at pulses of 0.3 seconds) and was centrifuged at 10,000 x g subsequently. The supernatant substance was cautiously removed and served for further processing; the pellet obtained in this step was discarded.

[0031] Subsequently to this, the solution was restricted in volume from 200 ml to 80 ml by means of an ultrafiltration ("Pellicon XL").

[0032] The protein solution thus obtained then underwent further processing by means of affinity chromatography (FPCL "Aktaexplorer").

[0033] The material containing the recombinant target protein was fractioned at 3 ml each, was applied and was fed over a column loaded with a metal-chelat matrix (NI-NTA, Qiagen) (volume : 8 ml)

This matrix specifically bonds the His tag of the recombinant protein.

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[0038] The protein solution thus obtained then underwent further processing by means of affinity chromatography (FPCL "Aktaexplorer").

[0039] The material containing the recombinant target protein was fractioned at 3 ml each, was applied and was fed over a column loaded with a metal-chelat matrix (NI-NTA, Qiagen) (volume : 8 ml)

[0040] This matrix specifically bonds the His tag of the recombinant protein. The target protein is retained by the metal and is washed under denaturing conditions (8 M of urea, 0.1 M of NaH_2PO_4, 10 mM of Tris/HCL, pH = 8).

[0041] After the contaminating proteins are removed the recombinant protein is desorbed by the affinity matrix by a pH leap (8 M of urea, 0.1 M of NaH_2PO_4, 10 mM of Tris/HCL, pH =3) and is collected at the exit of the column.

[0042] The purified protein is subjected to concentration by means of cross-flow filtration (pore size 5 kDa). After the purity and yield are checked (via an SDS gel electrophoresis, western blotting, Elisa, protein determination) the urea buffer is exchanged against a physiological buffer solution (PBS, pH = 7.2). Exchange is performed by means of cross-flow filtration (pore size 5 kDa).

[0043] The recombinant protein was present at a concentration of 300 ug/ml. * IPTG: Isoproplybeta-D-thiogalactopyranosite

Description of the recombinant fusion protein

[0044] The target protein is encoded by the gene fragment Stx2eB. The size of this fragment of the B sub-unit of Stx2e is 228 bp.

[0045] A test was made of the following properties of the recombinant protein:

1. Molecular weight size

The target protein has a molecular weight determined in the SDS gel electrophoresis of about 7.5 kDa.

2. Check of the recombinant protein in the Immunoblot with serums of fallen-ill piglets

The purified antigen was examined in the Immunoblot with serums of piglets fallen ill with the edema. The animals concerned were piglets from pig-breeding companies in which clinically manifest diseases occurred with Stx2e *E. coli* strains.

More than 90 % of these serums reacted positively with the recombinant protein. In order to exclude wrongly positive results, the examinations were verified with the B sub-unit coupled to the Glutathion S transferase of Schistosoma japonicum and were verified.

3. Check of the recombinant protein with monoclonal antibodies against Stx2eB

In order to find out whether the conformation of the recombinant B sub-unit is similar to the wild-type protein, the recombinant Stx2eB was examined with the Dot-Blot method. For this purpose, the monoclonal antibody BC5BB12 was used which specifically recognizes the B sub-unit of Stx2 and also cross-reacts with the B sub-unit of Stx2e. The Stx2e holotoxin was carried along as a positive check. A raw toxin preparation of StxI served as a negative check.

The monoclonal antibody BC5BB12 reacted with both the Stx2e holotoxin and the recombinant Stx2eB protein, but did not react with the StxI.

4. Test of the recombinant protein for cytotoxicity in the Verocell test

The cytotoxicity of the recombinant protein was tested on verocells, helacells, and MDBK cells in the cytotoxicity test. To this effect, concentrations of from 0.3 µg/ml to 100 µg/ml were employed on recombinant Stx2eB. Even in the lowest stages of dilution, no significant difference from the negative check was found to exist in any one of the cell lines examined. These results confirm that the recombinant Stx2eB is not cytotoxic per se.

5. Demonstration of the immunogeneity of the recombinant Stx2eB in the rabbit test

[0046] Two male rabbits of the "White New Zealander" race at the age of about 12 months were immunized with the recombinant Stx2eB. 100 µg of antigen were subcutaneously injected in the 1st vaccination while adding the incomplete Freund adjuvant (iFA).

[0047] Boostering was subcutaneously done with 50 ug of recombinant Stx2eB, also with iFA, six weeks later. The serums obtained prior to and after the vaccination were examined in the Immunoblot. A specific serum conversion was proved to exist in the two rabbits.

Description of how to prepare vaccine formulations (Examples)

1. How to prepare a W/O/W vaccine formulation:

The antigen is continuously added to the adjuvant (e.g. Montanide ISA 206) under sterile conditions as an aqueous phase (at a temperature of 22 °C) while being stirred (at a speed of < 2,000 r.p.m.). Subsequently, the emulsion is homogenized for 10 minutes at about 2,000 r.p.m. The vaccine formulation undergoes a new homogenization after a storage period of 24 hours at 8 °C. The phase position is tested microscopically and in a dyeing test.

2. How to prepare a W/O vaccine formulation:

The antigen is continuously added to the adjuvant (e.g. an incomplete Freund adjuvant) under sterile conditions as an aqueous phase (at a temperature of 22 °C) while being stirred (at a speed of < 2,000 r.p.m.) and is emulsified. The phase position is tested microscopically and in a dyeing test.

3. How to prepare an aqueous suspension:

The aqueous antigen is continuously added to the aqueous adjuvant (e.g. aluminum hydroxide) under sterile conditions while being stirred (e.g. using a magnetic stirrer) and is stirred. The vaccine is tested with respect to the pH and tonicity parameters.

4. How to prepare an O/W emulsion

The aqueous antigen is continuously added to the adjuvant and is emulsified. The phase position is tested microscopically and in a dyeing test. After the vaccine formulations are prepared they are stored in a refrigerator at temperatures of from +4 °C to +8 °C prior to their further use.

Example of how to prove the immunogenic action of the recombinant Stx2eB in the pig as a target animal by using various vaccine formulations

Object of the test

[0048] An examination is made on the question: Can the recombinant Stx2eB protein prepared by a genetic engineering method (using various adjuvants) induce an immunogenic response in the weaned piglet?

[0049] The test was made on 8 weaned piglets at the age of 6 weeks.

Six animals were treated with vaccine preparations, 2 animals were administered a placebo. The vaccine was applied twice at an interval of 3 weeks. Blood specimens were taken of each animal.

1. prior to the 1st immunization
2. 14 days after the 1st immunization
3. Directly prior to the 2nd immunization (21 days after the 1st immunization)
4. 14 days after the 2nd immunization
5. 21 days after the 2nd immunization

The serums were examined in the Elisa for the presence of specific antibodies which are directed against the recombinant Stx2 eB.

[0050] In addition, the compatibility and safety of the vaccines were assessed.

General testing data

Animals

Type of animal: Pig

Category of animal: Weaned piglet

Age: 6 weeks (at the time of 1st vaccination)

Sex: mixed

Immunity status of the animals at the start of tests: Stx2eB antibodies, negative

Way of keeping: in groups

Feeding scheme: ad libitum

Water supply: ad libitum from a water piping

Fodder additives used: no use of fodder additives

Vaccine administration parameters

Manner of application: by injection

Path of application: intramuscular

Period between the two vaccine applications: 3 weeks

Pre-treatment of the vaccine administered: nil

Pre-treatment of the animals being tested: nil

Number of animals being vaccinated: 8

Number of control animals: 2

Study design: randomized, blank

Vaccine dosage, animal identification, vaccine use

The definite test scheme is shown in Table 1.

Adjuvant key

Adjuvant A – ISA 206

Adjuvant B - iFA Adjuvant C -Montanide

Course of tests

[0051] Side effects encountered: After 1st vaccination- Slight effect on the general condition and fodder acceptance. Animals 7 and 8 showed a slight increase in body temperature + slight diarrhoea.

[0052] After 2nd vaccination- No further side effects, apart from increase in body temperature, for animal 8.

Number of animals which were withdrawn from the test:

Weaned piglet No. 2 because *E. coli* caused an intestinal inflammation.

Diseases which occurred, but were not due to the vaccination: None except for the disease of the weaned piglet No. 2

Treatment made with other medicines: nil

Results

Compatibility and safety of vaccine formulations:

[0053] The vaccine formulations can be considered to be generally compatible and safe although a slight disturbance of the general state of health, a short-time adverse effect on fooder acceptance, and an increase in the body temperature combined with

a slight diarrhoea occurred for the animals 7 and 8 after the 1st immunization. The 2nd immunization was stood with no appearance of clinical symptoms. Only the animal No. 8 reacted to the new vaccine application by an increase in body temperature.

[0054] Local tissue reactions - a slight edema detectable by palpation - only occurred at the injection point of animals 5 and 6 after the 1st vaccination.

[0055] After the piglets were slaughtered, macroscopically detectable inflammations were proved to exist at the points of injection around the injection channel, which were filled with necrotic material, except for the animals 1 and 3. When the piglets 1 and 3 were histologically examined only a slight connective-tissue proliferation (angioplasts with infiltered lymphocytes and histiocytes) was identified, whereas the injection channel filled with necrotic material was surrounded by a connective-tissue capsule in all of the other piglets. An inflammation with infiltered lymphocytes and histiocytes was observed in the connective-tissue capsule.

Effectiveness of the vaccine formulations:

[0056] This test demonstrated that the recombinant Stx2eB, after an intramuscular application in 6 weeks old weaned piglets, is identified by the immunity system of the animals and will induce an immunizing response, the production of specific immune globulins.

[0057] The existence of such antibodies was demonstrated by Elisa and Immunoblot.

[0058] The intensity of the immunizing response seems to be dependent on the choice of the vaccine formulation used.

[0059] The best results amongst the testing conditions chosen were achieved by a W/O emulsion (e.g. using iFA). The results are reported in detail in Table 2.

Table 1: Specimens tested, weight of animals, and the vaccine volume administered

No. of animals	Designation	Composition	Content of	Number of pigs /	Body weight in	Vaccine volume
1	Stx2eB Vaccine	PBS Adjuvant A		2	12,5	2,2
3	01 Placebo	Thiomersal			12,5	2,2
2	Stx2eB	rStx2eB	0,167 mg/ml	2	15	2,7
4	G97V27-02	Thiomersal			13	2,3
5	Stx2eB	rStx2eB	0,167mg/ml	2	16	2,9
6	G97V27-03	Thiomersal			12,5	2,2
7	Slx2eB	rStx2eB	0,250 mg/ml	2	14	2,5
8	G97V27-04	Thiomersal			14	2,5

Table 2:

Results of the serological examinations for Anti-S xB2e in the porcine serum specimens from the immunization test

Vaccine	Results for Immunoblot / ELISA (EL SA units)														
	27.1.1998			10.2.1998			17.2.1998			3.3.1998			10.3.1998		
	Nr.	IB	bLISA	Nr.	ID	hLISA	Nr.	IB	RLISA	Nr.	IB	ELISA	Nr.	IB	ELISA
Placebo adjuvant A	3	-	- (236)* + (403)	9 11	- -	- (357,6) - (356,3)	17 19	- -	- - (328)	2i 27	- -	- ?(1130	33 3<i	. .	-(398) -(380.3)
rStx2eB Adjuvant	2 4	- -	-(231) -(221)	10 12	/ -	/ - (350,3)	18 20	/ -	/ + (436)	/ 28	/ -	/ +(432,3	/ •<6	/ .	/ - (345)
rStx2eB Adjuvant B	5 6		(+) - (281.3)	13 14	++ (+)	+-H- + (432.6)	21 22	-M- +	+++ ++ (691.3)	29 JO	++ ++	+++ +++ (819.3)	37 38	++ +	+-H- +++ (1095.3)
rStx2eB Adjuvant C	7 8		- (233,6) -(241)	15 16		+ (474,6) + (425)	23 24	w (+)	+ (539,3) + (437)	31 32	+ "*	+ (473.6) + (411.6)	39 40	- -	+(411) -(389,3)

+ Positive check +++ (808); negative check - (187.3)

Deposited microorganism

[0060] The *E. coli* strain Cux-Stx2eB was deposited under the original designation Cux-SLT-IIe-B with the DSMZ - Deutsche Samml.ng von Mikroorganismen und Zellkulturen GmbH Mascheroder Weg Ibm D - 38124 Braur schweig. It was given the receipt No. DSM 12721 by the office of lodgement